Bromobimane crosslinking studies in oligomycin-sensitive ATPase from beef heart mitochondria

M_r 31000 protein crosslinked

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Using a bromobimane fluorescent label the M_r 31000 protein band oligomycin-sensitive (OS)-ATPase from beef heart mitochondria is shown to become much intensified by 2-mercaptopropionylglycine. In the presence of 3.5 nmol/mg protein of the thiol reagent ATP–P_i exchange activity is increased by 90%. With the fluorescent crosslinking reagent dibromobimane (DB) we show that a new fluorescent peak appears between M_r 50000 and 60000. ATP–P_i exchange is very much decreased by DB. The results suggest that for regulation of ATP-synthetase activity sulfhydryl groups in the region of the M_r 31000 protein(s) play an important role.

Mitochondrial OS-ATPase *Bromobimane labeling* *Crosslinking* M_r *31000 protein(s)*

1. INTRODUCTION

Discussion about the significance of the M_r 31000 protein(s) from oligomycin-sensitive (OS)-ATPase for ATP-synthetase activity is still controversial [1–3]. This heterogeneous protein band is present in some of the OS-ATPase preparations [1,3,4] but in others [2] it is absent. It has been claimed that ATP–P_i exchange still occurs, even in its absence [2]. Activity of the exchange, was, however, low compared to a preparation exhibiting a very prominent band at M_r 31000 [3].

Due to our crosslinking studies using diepoxybutane [5] it was suggested that dimers of the M_r 31000 protein band are formed, probably involving sulfhydryl groups. Since these dimers of M_r ~60000 were concealed in the bulk of α- and β-subunits of the OS-ATPase, they could not be shown in the gel electrophoresis after staining with protein reagent.

This paper attempts to clarify some of the intriguing aspects concerning the M_r 31000 protein(s) using bromobimanes [6] as sulfhydryl reagents.

2. MATERIALS AND METHODS

2.1. *Isolation of beef heart mitochondria*

Beef heart mitochondria were isolated and submitochondrial particles were prepared as in [4,7]. Protein was determined according to [8].

2.2. *Preparation of OS-ATPase*

OS-ATPase was prepared according to [4], omitting the sucrose gradient step. The final pellets of the ATPase were suspended in a buffer containing 10 mM triethanolamine–HCl, 0.4 mM EDTA, 1.0 mM $MgSO_4$, and 50 mM sucrose, adjusted with KOH to pH 7.5 and stored at −80°C; protein was 30–40 mg/ml.

2.3. *Fluorescent labeling using monobromobimane (MB)*

Protein (2.5 mg) was incubated in 500 μl final vol. of 10 mM Tris–sulfate (pH 7.5), 0.5 mM EDTA, 1.0 mM $MgSO_4$, 50 mM sucrose at pH 7.5 and 20°C. After 2 min 30 μM (1.5 μl of a 10 mM aqueous solution) of 2-mercaptopropionylglycine

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(MPG) was added, 1 min later 10 μM of monobromobimane (MB) (0.83 μl of a 6 mM solution in acetonitrile). The same amount of acetonitrile was also added to the controls without MB. Thereafter incubation at 20°C was done for 30 min. Subsequently, 20 mg sodium dodecylsulfate (SDS), 100 μl 0.5 M NaP_i (pH 7), 100 μl glycerol and 15 μl mercaptoethanol was added to the samples. The material (25 μl) was layered on top of the slab gels for electrophoresis.

2.4. *Fluorescent labeling with the crosslinking reagent dibromobimane (DB)*

The incubation was similar to the procedure above under MB labeling. In cases, where *N*-ethylmaleimide was added (2.5 μl of a 10 mM aqueous solution = 300 μM) the preceding incubation at 20°C was 2 min. Specificity of the bromobimanes for sulfhydryl groups is indicated by the fact that after pretreatment of the ATPase with *N*-ethyl-maleimide and subsequent treatment with DB nearly no fluorescence appeared on the gels (not shown). After 1 min, dibromobimane (2.5 μl = 300 μM, dissolved with acetonitrile) was added. Controls were treated with the same amount of acetonitrile. After further incubation for 30 min, additions were carried out similar as above under MB labeling, to prepare the samples for electrophoresis.

2.5. *SDS–Polyacrylamide gel electrophoresis*

Gradient slab gel electrophoresis was performed with 5–15% gels. Electrode buffer: 50 mM Tris, 0.38 mM glycine, 0.1% SDS, 2 mM EDTA (pH 8.8). About 80–100 μg protein was applied to the gels. Electrophoresis was carried out at a constant current of 30 mA, for ~2 h. Fixation, staining and destaining of the gels was performed in the same system of 25% methanol, 10% acetic acid, 65% H_2O. Serva Blau R Puriss 0.05% was added to that system for staining.

2.6. *Scanning of fluorescent and of protein-stained bands*

We used a Zeiss spectrophotometer M4QIII, equipped with a SP 4100 computing integrator, Spectra Physics (Santa Clara CA). The light path was arranged for remission measurement technique at an estimated angle of 90°. The gels were placed between two glass plates and scanned with a drive of 2 cm/min.

(i) The unstained but bromobimane-labeled gels were excited with monochromator-filtered light at 385 nm and the emitted light was filtered with a cut-off filter at 430 nm.
(ii) The subsequently stained gels were irradiated with monochromator-filtered light at 560 nm and the remitted light was measured.

2.7. *Estimation of ATP–P_i exchange activities*

$ATP–P_i$ exchange activities were measured following [4] as modified [9]. Finally we added to the incubation medium 1.0 mM ATP and 0.5 mM ADP together with P_i and ^{32}P as in [3].

2.8. *Substances*

MB and DB were obtained from Calbiochem (Giessen), ^{32}P (10 mCi/ml diluted HCl) was purchased from Buchler-Amersham (Braunschweig).

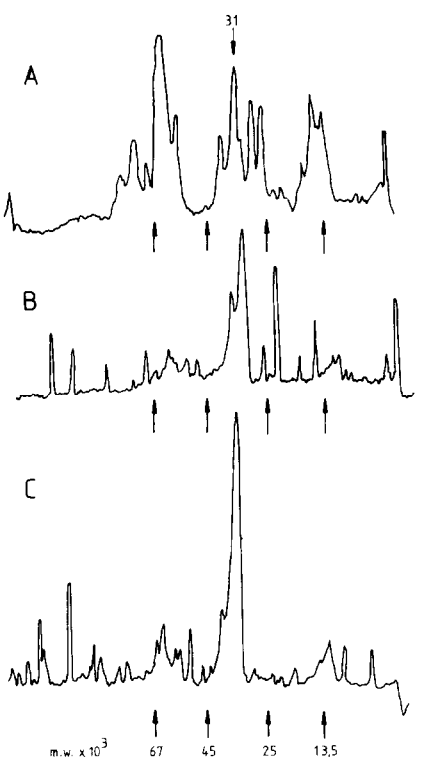

Fig. 1. Monobromobimane fluorescence of OS-ATPase: (A) protein-stained control experiment measured at 560 nm; (B) fluorescence of the gel shown above, 10 μM MB; (C) fluorescence of a gel from OS-ATPase in presence of 30 μM MPG, thereafter 10 μM MB was added.

3. RESULTS

Results obtained with monobromobimane are presented in fig. 1. We find that particularly protein of M_r 29–31 × 10^3 is labeled. Moreover, some label is also found at M_r 50–60 × 10^3 and M_r 7–15 × 10^3. In presence of 30 μM 2-mercaptopropionylglycine these peaks become intensified (fig. 1C).

Crosslinking the OS-ATPase by 300 μM of dibromobimane is shown in fig. 2. Fluorescence now appears mainly at M_r 50–60 × 10^3, while the fluorescence in the M_r 29–31 × 10^3 protein is very much decreased. This indicates the crosslinks have been formed between proteins of M_r 29–31 × 10^3. This dimerization becomes even more evident in presence of 0.5 mM ATP: The rest of fluorescence in the M_r 29–31 × 10^3 region nearly vanishes while the peak at M_r 50–60 × 10^3 appears somewhat broadened (cf. fig. 2B with D, fig. 2C with E).

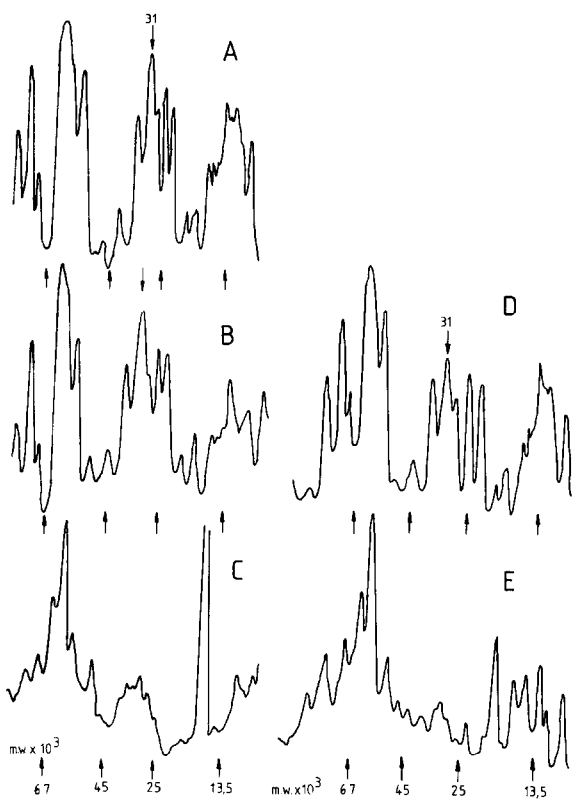

Fig. 2. Dibromobimane fluorescence of OS-ATPase: (A) protein-stained control experiment measured at 560 nm; (B) protein-stained experiment in presence of 300 μM DB; (C) fluorescence of (B); (D) protein-stained experiment after addition of 0.5 mM ATP to the OS-ATPase, thereafter 300 μM DB was added; (E) fluorescence of (D).

Table 1

ATP–P_i exchange activities of OS-ATPase

Addn.	[Reagent]	% Control activity[a]
nil		100
MB	10 μM	98
MB	300 μM	34
DB	300 μM	37
Oligomycin	10 μg/mg protein	29
FCCP	1 μM	34
MPG	1 nmol/mg protein	139
MPG	2 nmol/mg protein	171
MPG	3.5 nmol/mg protein	193
MPG	7 nmol/mg protein	93
MPG	14 nmol/mg protein	101

[a] Control activity was 235 $nmol.min^{-1}.mg\ protein^{-1}$

The intense fluorescent peak at M_r ~15000 is, presently, not interpretable. Though no protein counterpart is visible, nevertheless a non-stainable protein might exist.

Table 1 exhibits the results obtained for ATP–P_i exchange experiments in presence of different reagents. While 10 μM of MB did not alter the exchange rate, 300 μM of DB and MB as well resulted in significant decreases of the exchange. Similar decreases were observed in presence of oligomycin and FCCP. MPG, by contrast, increased the exchange rate by ~40–90% in a concentration-dependent manner.

4. DISCUSSION

By means of the fluorescent SH-reactive bromobimanes we obtain evidence for reaction with protein at M_r 29–31, 50–60 and 7–15 × 10^3. Changes in these regions of the OS-ATPase molecule may be interrelated. With MB, fluorescence is most prominent in the M_r 29–31 × 10^3 protein(s). This domain as well as that in the low-M_r range, became increased in protein-staining intensity by MPG [7,10]. It was also suspected that protein of M_r 29–31 × 10^3 could dimerize to M_r 50–60 × 10^3 [5].

However, this never could be positively demonstrated, because of the high amount of protein in the α-, β-region of the ATPase.

Using the bifunctional label DB, we are now able to show high fluorescence intensity at M_r $50–60 \times 10^3$, and only low fluorescence in the M_r $29–31 \times 10^3$ range. This rest intensity could become further diminished by 0.5 mM ATP. It is known, that ATP-Mg^{2+} (ADP) unmasks reactive SH-groups [11–13] which probably are interrelated with the adenine nucleotide translocase [14]. However, uncoupler binding protein(s) [15] and P_i translocator [16,17] may contribute here. Further work on the interrelations and composition of the heterogeneous protein band at M_r $29–31 \times 10^3$ is necessary.

This is the first direct evidence for dimerization of the M_r $29–31 \times 10^3$ protein(s) to M_r $50–60 \times 10^3$ within the OS-ATPase complex.

ATP–P_i exchange further underlines the importance of SH-groups for ATP-synthesis: While in presence of the bromobimanes the exchange activity decreased to ~35% of the control activity, with the thiol reagent MPG we obtained increases of the exchange by ~40–90%.

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